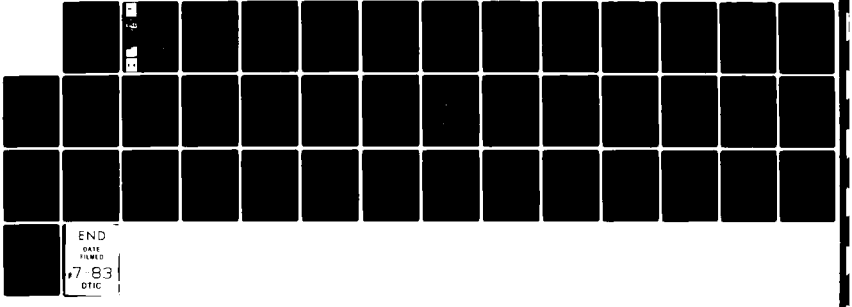
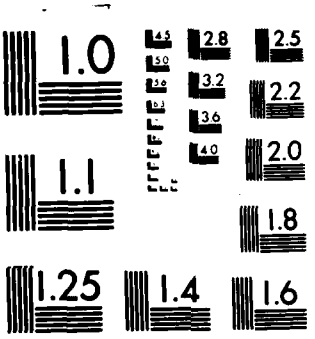
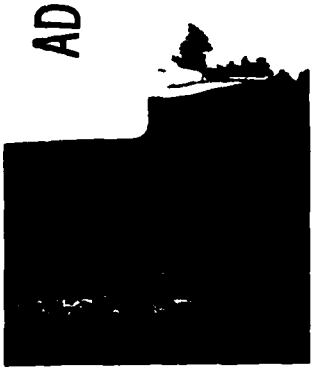
AD-A129 105 STUDY OF A NATURALLY OCCURRING HYDRILLA INHIBITOR(U) 1/1
UNIVERSITY OF SOUTH FLORIDA TAMPA CHEMICAL AND
ENVIRONMENTAL MANAGEMENT CENTER D F MARTIN MAR 83
UNCLASSIFIED WES-MP-A-83-2 DACW39-80-C-0066 F/G 13/2 NL



END
DATE FILMED
7-83
DTIC



US Army Corps of Engineers



AQUATIC PLANT CONTROL RESEARCH PROGRAM

MISCELLANEOUS PAPER A-83-2

STUDY OF A NATURALLY OCCURRING HYDRILLA INHIBITOR

by

Dean F. Martin
Chemical and Environmental Management Center
University of South Florida
Tampa, Fla. 33620

March 1983
Final Report

Prepared for Office, Chief of Engineers, U. S. Army
Washington, D. C. 20314
Under Contract No. DACW39-80-C-0066
Monitored by Environmental Laboratory
U. S. Army Engineer Waterways Experiment Station
P. O. Box 631, Vicksburg, Miss. 39180

Destroy this report when no longer needed. Do not return it to the originator.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

The contents of this report are not to be used for advertising, publication, or promotional purposes. Citation of trade names does not constitute an official endorsement or approval of the use of such commercial products.

The covers of U. S. Army Engineer Waterways Experiment Station (WES) engineering and scientific reports have been redesigned. Each WES Laboratory and support organization will have its own distinctive color imprinted on white coverstock. This standardizes WES publications and enhances their professional appearance.

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Miscellaneous Paper A-83-2	2. GOVT ACCESSION NO. A129105	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) STUDY OF A NATURALLY OCCURRING HYDRILLA INHIBITOR		5. TYPE OF REPORT & PERIOD COVERED Final report 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Dean F. Martin		8. CONTRACT OR GRANT NUMBER(s) Contract No. DACW39-80-C-0066
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of South Florida Chemical and Environmental Management Center Tampa, Fla. 33620		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Aquatic Plant Control Research Program
11. CONTROLLING OFFICE NAME AND ADDRESS Office, Chief of Engineers, U. S. Army Washington, D. C. 20314		12. REPORT DATE March 1983 13. NUMBER OF PAGES 36
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) U. S. Army Engineer Waterways Experiment Station Environmental Laboratory P. O. Box 631, Vicksburg, Miss. 39180		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Available from National Technical Information Service, 5285 Port Royal Road, Springfield, Va. 22151.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Aquatic plant control	Lake Starvation
Aquatic weeds	Sediment
Hydrilla	Weed control

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An investigation was made of sediment from Lake Starvation (northwest Hillsborough County, Florida) where hydrilla, though present, has not spread during a 10-year period. The sediment was treated with water, autoclaved, and the aqueous extracts separated into molecular weight fractions by ultrafiltration. One fraction, thus obtained, inhibited the growth of hydrilla by 30 percent, as measured by the weight increase, relative to control samples. The active fraction was characterized by inorganic and organic content, as well as

(Continued)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. ABSTRACT (Continued).

trace metal content (iron, manganese, nickel, and cadmium).

Subsequently, the active fraction was separated further using high-performance liquid chromatography, and the components thus separated were characterized by ultraviolet absorption spectra and by their effect on the size distribution of *Chlamydomonas reinhardii*.

One component that showed significant bioactivity according to this assay was subjected to mass spectrometry. The maximum molecular ion for this component was less than 500. The available evidence is consistent with an aromatic molecule that contains a carboxylic acid. Further evidence includes the infrared spectra and the observation that all activity was lost when the sample was passed over an anion-exchange resin, whereas no activity was lost when the sample was passed over a cation-exchange resin.

Preface

This report presents the results for FY 80 of an ongoing study to discover and evaluate naturally occurring chemicals that inhibit the growth of *Hydrilla verticillata* (Royle) and to provide information that will assist in the evaluation of these materials as aquatic plant herbicides. The project is being conducted for the Aquatic Plant Control Research Program (APCRP) by the Chemical and Environmental Management Service (CHEMS) Center, Department of Chemistry, University of South Florida, Tampa, Fla., under Contract No. DACW39-80-C-0066. Funds for this effort are provided by the Office, Chief of Engineers, U. S. Army, under appropriation number 96X3122 Construction General (902-740) through the APCRP at the U. S. Army Engineer Waterways Experiment Station (WES), Vicksburg, Miss.

The principal investigator for the work was Dr. Dean F. Martin, CHEMS, who prepared this report in cooperation with members of the CHEMS Center. Technical assistance was provided in the CHEMS Center by Vance Ley, Julio Herrera, Ralph Moon, Mark J. Halvorson, Robert Chatham, Patricia M. Dooris, Sandra L. Fisher, Barbara B. Martin, and Louise B. Worrell.

The work was monitored at WES by Dr. Howard Westerdahl, Environmental Laboratory (EL), WES. Mr. J. Lewis Decell was Manager, APCRP. Dr. John Harrison was Chief, EL.

Commander and Director of WES during preparation of this report was COL Tilford C. Creel, CE. Technical Director was Mr. F. R. Brown.

This report should be cited as follows:

Martin, D. F. 1983. "Study of a Naturally Occurring Hydrilla Inhibitor," Miscellaneous Paper A-83-2, prepared by University of South Florida, Tampa, Fla., for the U. S. Army Engineer Waterways Experiment Station, CE, Vicksburg, Miss.

Contents

STUDY OF A
NATURALLY OCCURRING HYDRILLA INHIBITOR

Introduction

Background

1. Noxious aquatic plants can interfere with the availability of freshwater supplies for agricultural uses and for navigation in Florida and other areas of the United States, as well as Panama and India. Nuisance growth of certain aquatic plants affects the flow and use of water for irrigation and domestic use. Navigation is impaired and economic losses can be severe when an aquatic plant such as hydrilla (*Hydrilla verticillata* Royle) invades a lake, river, or canal, particularly if the water body has recreational uses.

2. Although management of aquatic plants is mainly accomplished through the use of herbicides, the number of suitable compounds is limited. Federally registered herbicides for hydrilla control include endothal, diquat, 2,4-D (2,4-dichlorophenoxyacetic acid), and various formulations of chelated copper sulfate. The increasing cost of petroleum is likely to increase the costs of herbicides derived from petroleum.

3. Thus, there is a critical need to find environmentally acceptable herbicides for use in aquatic herbicides through discovery of naturally occurring inhibitors of plant growth.

Purpose and Scope

4. The purpose of this research was to investigate some chemical and biological characteristics of aqueous extracts of a sediment from Lake Starvation because these extracts are known to inhibit the growth of hydrilla in the laboratory. The extent of inhibition was

investigated; the chemicals were separated into fractions; and the hydrilla-inhibiting activity of the fractions was investigated.

Materials and Methods

Source of lake sediment

5. Lake Starvation is located in northwest Hillsborough County, Florida (Figure 1). The lake is 15.0 m above mean sea level, and the contour map (Figure 2) shows that it is a shallow lake with gently sloping sides (Dooris and Moresi 1975). The soils surrounding this lake were originally part of a freshwater swamp with the major overstory composed of *Taxodium distichum* (bald cypress) (Soil Conservation Service 1958). The lake is situated in a major well field for St. Petersburg, Fla., and it has been developed into a day-use park with boating facilities. Enrichment from point-source pollution is not a problem in this lake, but the lake has been artificially augmented with water from the Floridan Aquifer since September 1972 using wells that have an augmentation capacity of about 3800 ℓ/min. As a result of augmentation, the lake area has doubled from a historical low of 10.1 ha to 20.2 ha (Dooris and Martin 1979).

Preparation and analysis of lake sediment extracts

6. Sediments from a littoral zone of Lake Starvation (Figure 2) were collected and sent on ice to the laboratory. Extracts of each sediment sample were prepared according to the following scheme. Fresh wet sediment (300 g) was subjected to autoclaving (120°C, 138 kPa, 20 min) in the presence of 600 ml of deionized-distilled water. The resulting dark-colored fluid was separated from the sediment by filtration (Figure 3), first through Whatman No. 1 filters, followed by Millipore 8-μ filters. An aliquot of the filtrates (Fraction A) was reserved and subjected to no further filtration. The remaining filtrate was subjected to ultrafiltration and analysis (Table 1).

Preparation of other extracts

7. Humic acid extract. A 150 g sample of humic acid (Aldrich) was treated with 300 ml of deionized-distilled water and autoclaved

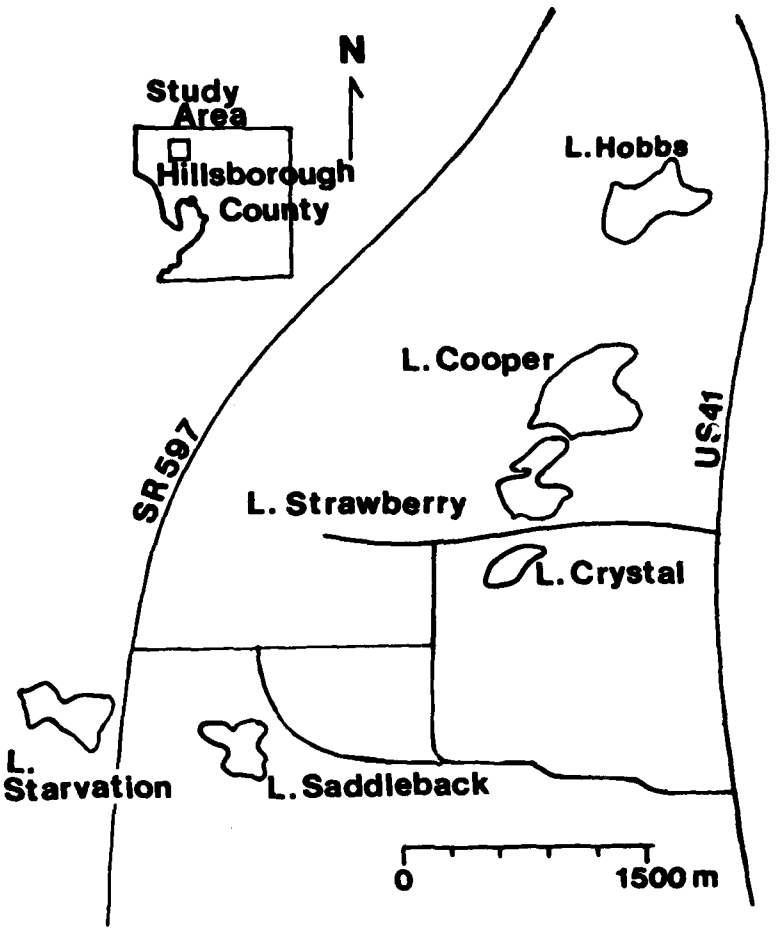


Figure 1. Map of study area, Hillsborough County, Florida (after Dooris and Moresi 1975)

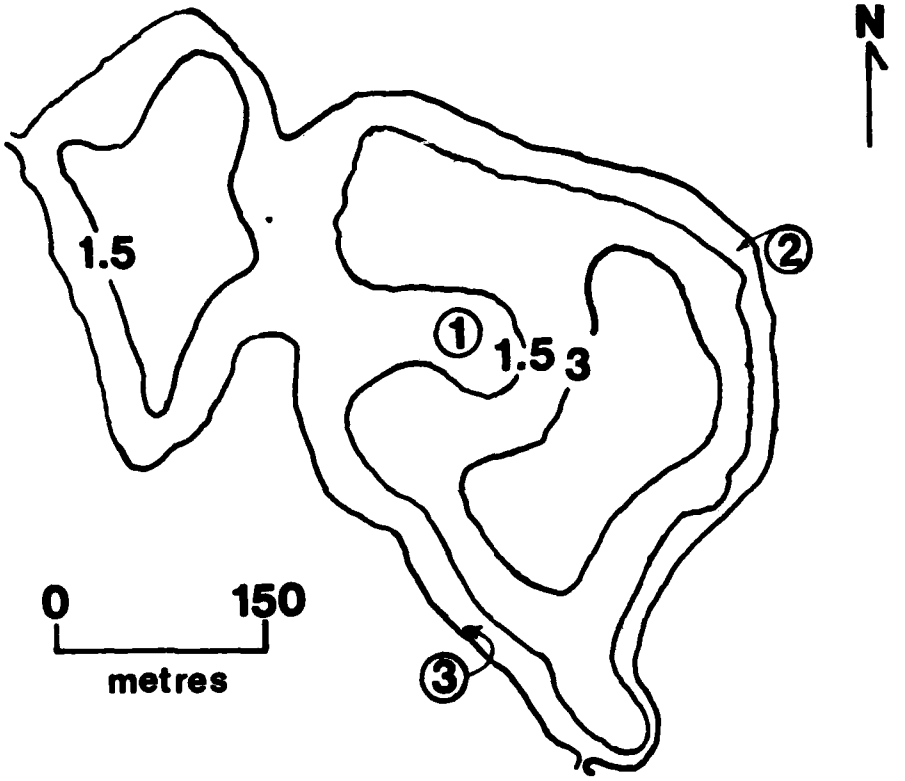


Figure 2. Contour map of basin of Lake Starvation. Contour lines (in metres) indicate depth-to-bottom contour below normal lake stage (15 m above sea level). Substrate at stations: 1, clay; 2, peat; and 3 (sampling site), peat/organic (after Dooris and Moresi 1975)

(120°C, 138 kPa, 20 min). The resulting dark fluid was separated from the solid by filtration (Whatman No. 1, followed by 8-μ membrane filter). An aliquot portion was reserved for analysis (Fraction A, Figure 3), then the remainder was subjected to ultrafiltration.

8. Sodium humate extract. A 15-g sample of sodium humate (humic acid, sodium salt, Aldrich) was dissolved in 600 ml of redistilled deionized water, autoclaved, and subjected to the same filtration and ultrafiltration procedures as above.

9. Room temperature extraction of sediment. Lake Starvation sediment (150 g in 300 ml of distilled water) was placed in a nitrogen-filled stoppered flask and agitated in a gyrotary water bath shaker (model G 76, New Brunswick Scientific) at room temperature (ca. 25°C) for 68 hr. The extract obtained was subjected to the usual filtration procedure, and the fractions collected were analyzed for carbon content (Table 2).

Ultrafiltration

10. An Amicon filtration apparatus, operated at 280 kPa nitrogen, was used with um-10, um-2, and um-05 filters with nominal molecular weight cut-off points at 10,000, 2,000, and 500, respectively. A portion of each filtrate was reserved and the <10,000 material was referred to as Fraction B; the <2,000 material as Fraction C; the <500 material as Fraction D (Figure 3). The extracts were stored in tightly closed glass bottles at -30°C.

Analysis of sediment, water, and organic carbon

11. Representative samples of Lake Starvation sediment were obtained by sectioning and comingling. Water content was determined by loss of weight following drying at 110 ± 5°C for 18 days. Organic carbon (Table 1) was determined as weight loss after heating in a muffle furnace 16 hr at 375°C (Ball 1964).

Manganese and iron content

12. Sodium acetate extraction. A sodium acetate extract of Lake Starvation sediment was dried (electric oven, ca. 105° ± 5°C) to constant weight and samples (ca. 0.5 g) were ground and washed. Digestion was accomplished by heating the samples in 30 percent H_2O_2 with sodium

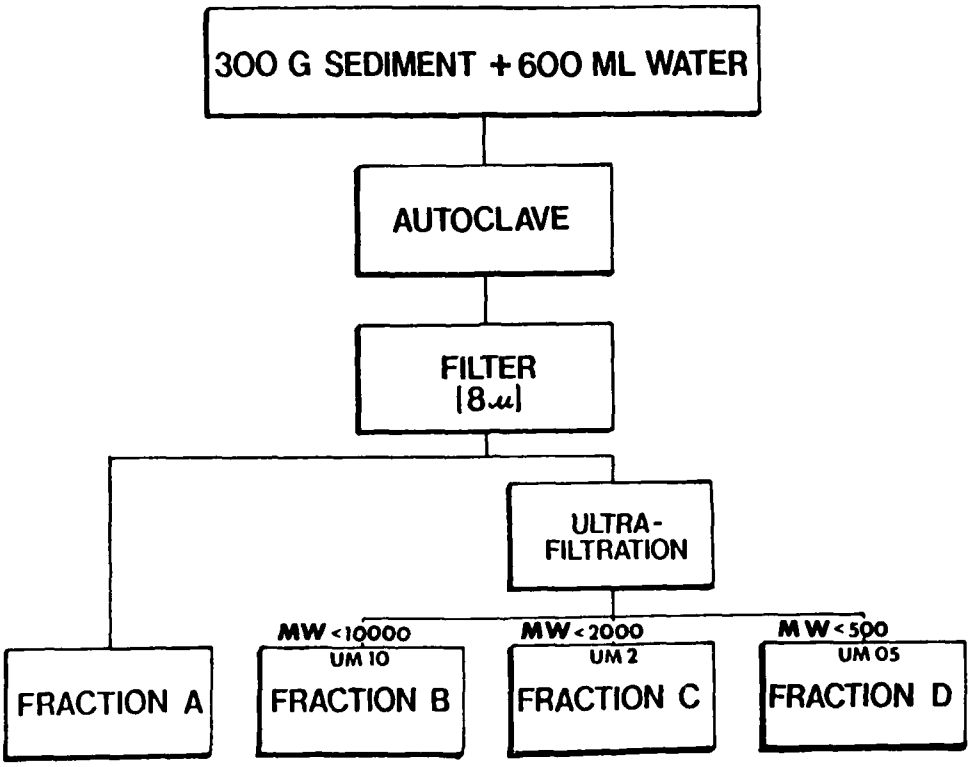


Figure 3. Summary of procedures used to extract lake sediment, commercial humic acid, and sodium humate (MW = molecular weight)

acetate buffer (pH 5) until only sandlike material remained (Jackson 1956). A blank sample was also prepared. The digested samples were filtered (Whatman No. 1) and diluted to 100 ml.

13. Ammonium acetate extraction. Six ammonium acetate extraction replicates of Lake Starvation sediment and a blank were prepared. Digestion was accomplished by heating the samples in 10 ml of 30 percent H_2O_2 which had been made acidic with HCl (pH 2.5) (Chen et al. 1976). The samples were heated to near dryness, at which point additional aliquots of 10 ml of 30 percent H_2O_2 (pH 2.5) were added. The above procedure was repeated until two successive additions and evaporations showed no evidence of reaction (vigorous foaming) in any of the samples. Seven additions of the H_2O_2 solution were required. The samples, evaporated almost to complete dryness, were extracted in 100 ml of 1 M ammonium acetate (pH 2.5 with HCl) for 1 hr on a shaker. The resulting solutions were filtered (Millipore membrane, 0.22 μm) and analyzed by atomic absorption spectroscopy (Table 1). All standards were prepared in ammonium acetate solution (1 M pH 2.5). Samples of aqueous extracts were analyzed for Fe and Mn using atomic absorption spectroscopy (Perkin-Elmer 603, air-acetylene flame). Results are presented in Table 1.

Separation of fractions by high performance liquid chromatography (HPLC)

14. Equipment and techniques. High performance liquid chromatography (HPLC) on Lake Starvation sediment extracts was carried out on an Altex (Model 110A) liquid chromatograph with solvent programmer attachment (Model 1601) and an ultraviolet (UV) detector (Model 153, 254 nm). Separation of the sediment extract was achieved using a reverse-phase column (Altex Ultrasphere, ODS, 5-μ particle size). Several gradient systems were tested (see Table 3) and different programs were run (i.e., different settings of length of run, different initial and final mixtures of methanol-water, different patterns of going from initial to final mixtures of solvents). Programs 2 to 3 (Table 1) appeared to provide optimum component resolution.

15. Sep-Pak modification. A C_{18} reverse-phase chromatography

cartridge (Sep-Pak, Waters Associates) was used for rapid sample preparation and to remove any problems caused by components tenaciously adhering to the HPLC analytical column. The cartridges were first wet with 2 ml of redistilled methanol, then washed with 10 ml of deionized water redistilled (from permanganate). Next the sample was passed through the activated cartridge using a syringe (ca. 0.5 ml/min). Total carbon analysis and examination of the cartridge indicated that some material was adsorbed, although more than 90 percent of the carbon was eluted from the humic acid extract. Additional colored material was removed by elution with methanol, but some material still remained on the cartridge. Both the aqueous and methanol eluants were subjected to separation by HPLC. Water used was HPLC grade.

16. Fractionation of extracts. Extracts were injected into the chromatograph using a 20-µl injection loop. Following the elution of all peaks, elution was continued to ensure that no other peaks could be detected. Each injected sample was monitored for aromatic and other compounds absorbing at 254 nm, and all fractions were collected in test tubes. Individual samples were reduced in volume by solvent evaporation using a Buchi-Rotavapor at 41°C and 75 torr, or at room temperature in a stream of nitrogen.

Spectra

17. Infrared spectra were obtained using a Perkin-Elmer grating infrared spectrophotometer (Model 337). A sample was prepared by dissolving the extract residue in acetonitrile and evaporating the solution on a sodium chloride window. Ultraviolet spectra were obtained as acetonitrile solutions using a Carey 14 spectrometer.

Growth experiment

18. Procedures. Hydrilla was harvested from a nearby lake (Lake Saddleback, Figure 1) or river (Anclote). Plants were rinsed in tap water for 1 day, then rinsed in deionized water for 1 day. Studies were conducted using stoppered, inverted 500-ml Erlenmeyer flasks as growth vessels. Each of the 10 to 15 control vessels contained a known length (75 mm) and mass (approximately 2 to 3 g) of apical plant material submerged in a 10 percent Hoagland's solution (Steward and

Elliston 1973) which contained 5 ppm inorganic carbon as $KHCO_3$. The 10 to 15 test vessels were supplemented with 0.8 ml of each fractionated water extract in addition to the nutrient solution. Flasks were placed in the Phytotron and illuminated 14 hr/day (75 to 100 μeinsteins/m^2/sec, as measured by a Li-Cor model 185 photometer) using rows of fluorescent lamps mounted in the ceiling. Tests were discontinued at the end of 7 days. Growth estimates were based on changes in wet weight (as measured to ± 0.0001 g) during the same period and had been evaluated previously. The precision of the wet-weight measurements was good with a relative standard deviation of 0.34 percent for ten measurements (Dooris and Martin 1980). In addition, dissolved oxygen, inorganic carbon, and pH were measured for the control and test solutions at the end to the study.

19. Modifications. In some designated experiments, an algicide was used. Hydrilla was soaked in 0.01 percent (by weight) aqueous $HgCl_2$ for 15 sec then thoroughly rinsed in distilled water for 30 sec (Steward and Elliston 1973). In another modification, the effect of sediment was tested, and 60 ml of an equal weight mixture of Michigan peat and washed builder's sand was used in the study flasks (Cooley, Dooris, and Martin 1980). Studies were continued for a 2-week period. The mean weight increase for control samples was 3.5 ± 1.7 percent, and for the test samples with extract (0.4 ppm organic carbon) was -16.2 ± 2.9 percent.

20. In order to obtain as uniform a population of hydrilla as possible, a culture vessel was required that would continually circulate the plants. By providing this circulating effect, variations in the population due to shading and depth may be eliminated.

21. The vessel was constructed by dividing a 366-ℓ Plexiglas tank (73 cm x 83.5 cm x 60 cm) with a sheet of Plexiglas on a diagonal from the top of the tank to the bottom (Figure 4). A polyvinyl chloride tube (1.3 cm diam), which had a large number of small holes (No. 60) drilled along the length, was placed along the bottom edge of the tank so that it rested on top of the aforementioned diagonal sheet to Plexiglas. One end of the tube was stoppered, and the other end was connected via

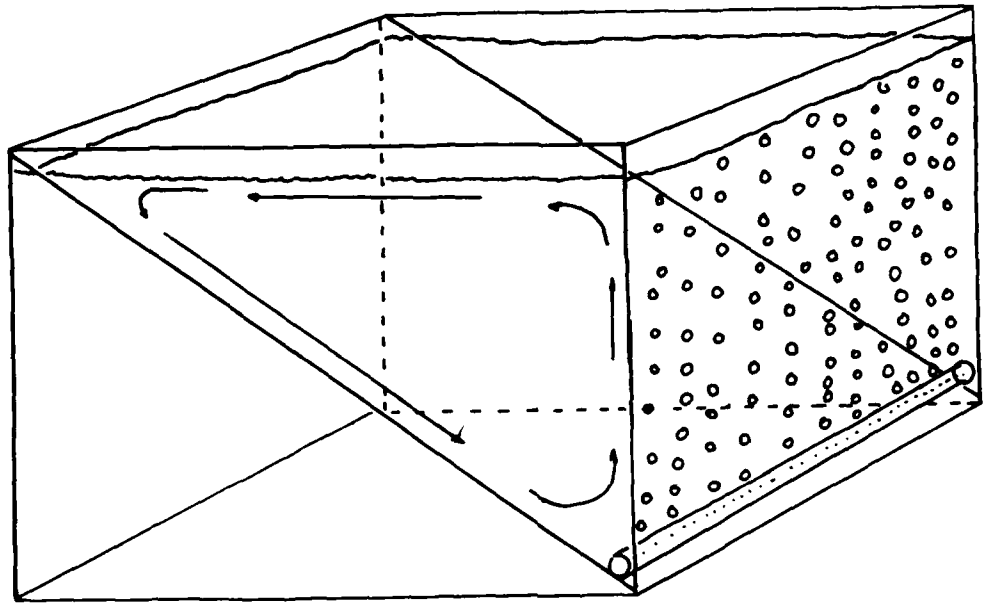


Figure 4. Hydrilla culture tank

a Tygon tube to a compressed air supply. The compressed air exited the tube through the small holes, and the bubbles created a convection current of sufficient intensity to cause the plants to circulate through the upper half of the tank. The air flow was adjusted to provide a turning rate that was not damaging to the plants. The water used was obtained from the University of South Florida (USF) Science Center's well (Floridan Aquifer). Sixteen litres of the water was changed daily.

Use of alga in assay
of hydrilla inhibitor

22. Slant cultures of the unicellular green alga *Chlamydomonas reinhardii* were obtained from Dr. Allan Michaels, Department of Biology, USF. An appropriate inoculum was added to a medium (Table 4), which had been filtered previously through 0.22-μ membrane filters and autoclaved. Samples of the alga were obtained and tested for precision of counting by a Coulter electronic particle counter (Model ZB_I with a C-1000 Channelyzer attachment). Settings with a 100-μ aperture were:

amplification, 1; aperture current, 0.354; lower threshold, 20; upper threshold, 90; count control, timer, 10 sec. The samples were diluted with Isoton counting solution, and the results of successive dilutions are summarized in Figure 5 and Table 5. Knowing the median volume of the cells in threshold units (Figure 5), the median volume may be calculated from the relationship:

$$K = V/IAT$$

where

K = calibration constant, obtained with standard spheres

V = the median cell volume, μm^3

I = aperture current

A = amplification

T = median cell volume, in threshold units, as obtained from Figure 5

A median cell volume of about 170 μm^3 was obtained. The cells were counted with good precision using a timed-flow technique (e.g., the number of cells are counted during a given time period, rather than by sample size). The results listed in Table 5 indicate that there is a linear relationship between the number of cells and the relative dilution over a fair range of cells. For example, between 5,000 and 30,000 cells, the number of cells is a linear function of the relative number of cells ($r = 0.9955$; $P < 0.01$), and the correlation was even better between 5,000 and 17,000 cells ($r = 0.9999$; $P \leq 0.001$). In addition, previous work in this laboratory has demonstrated that there is a direct relationship between the number of cells counted during a 10-sec flow and the cells counted for a 0.5-ml sample. Better precision is obtained for the timed-flow method, however.

Mass spectral analyses

23. Significant fractions collected from HPLC separations were collected and reduced to dryness under nitrogen. Mass spectral analyses were performed at the Florida State University mass spectrometry laboratory to determine the major ion fragments, using either electron impact or chemical ionization spectrometry in the range of m/e 0 to 650.

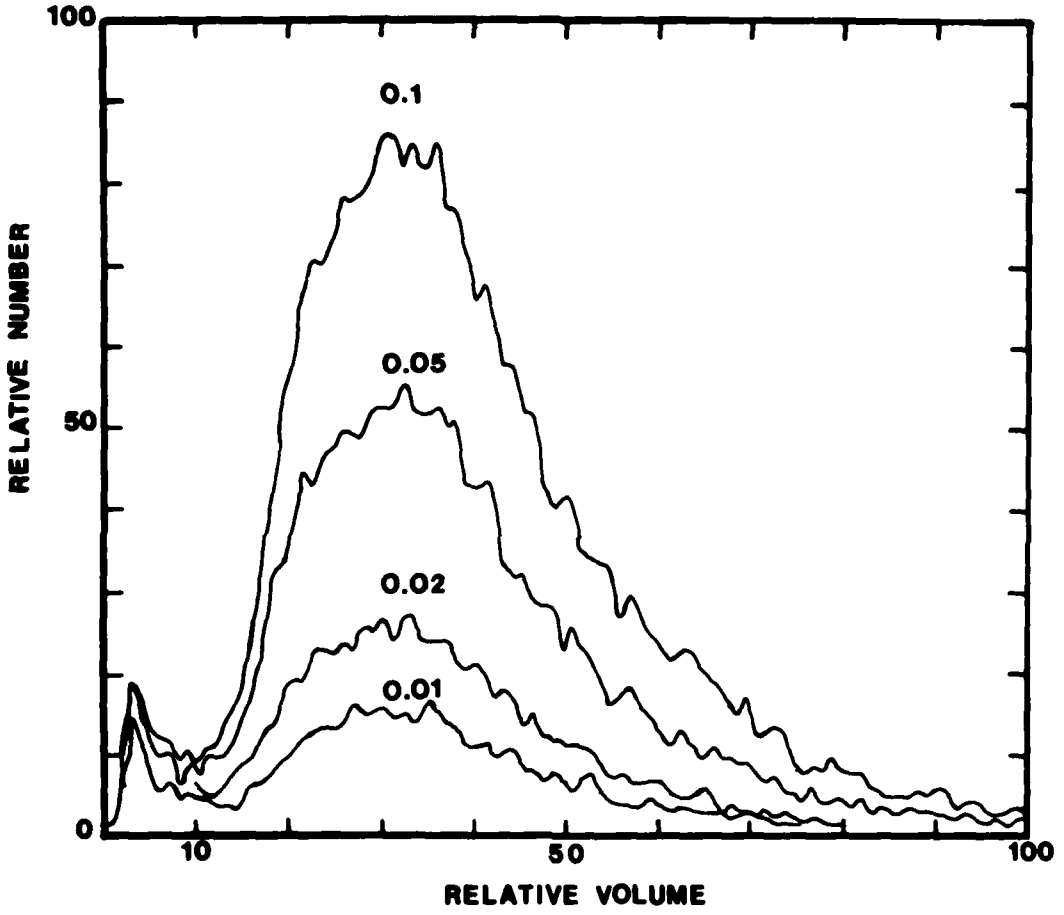


Figure 5. Relative cell number as a function of relative volume (expressed in threshold units) of *Chlamydomonas reinhardii* at dilutions indicated (settings: amplification, 1; aperture current, 0.354; 10-sec flow using a Coulter Model ZB_I)

Results and Discussion

24. The sediment was analyzed for iron and manganese content because these elements are known to affect the growth of hydrilla (Reid, Martin, and Kim 1975). The concentration of manganese in the sediment and in the aqueous extracts was less than the sensitivity of the analytical method (10 ppb). The iron content in the sediment (Table 1) was moderate, and the content in the parent aqueous extract (Fraction A) was about 550 ppm.

25. The possibility that the inhibitory effects of Lake Starvation extracts were the result of the heavy metal content cannot be eliminated at this stage, but two observations are pertinent. Inhibition was observed for solutions containing dilute extracts (0.4 ppm as organic carbon) and the iron content in those solutions was less than 0.09 ppb (by extrapolation). Previous results (Reid, Martin, and Kim 1975) indicated an inhibitory effect of iron, but the value was above 150 ppb (as chelated iron); below that value, the iron was stimulatory.

Lake Starvation sediment fractionation

26. The crude extract was fractionated using ultrafiltration into nominal molecular weight fractions. From the parent fraction, additional fractions (Figure 3) were obtained using filters. Fraction B_1 (i.e., the material that passed through the <10,000 filter but not the <2,000 filter) had maximum activity with respect to inhibition of hydrilla growth. Fractions C_1 and D_1 either had much less inhibitory activity or did not differ significantly from control experiments (Figure 6).

27. In addition, a further separation was made for purposes of analyzing the samples by means of HPLC. The fractionated sample (B_1) was adsorbed onto a short (13 x 7 mm) methanol-activated C-18 reverse-phase liquid chromatography column (Sep-Pak), and the eluant was collected. In addition, the methanol-soluble fraction was also collected. Both were subjected to fractionation.

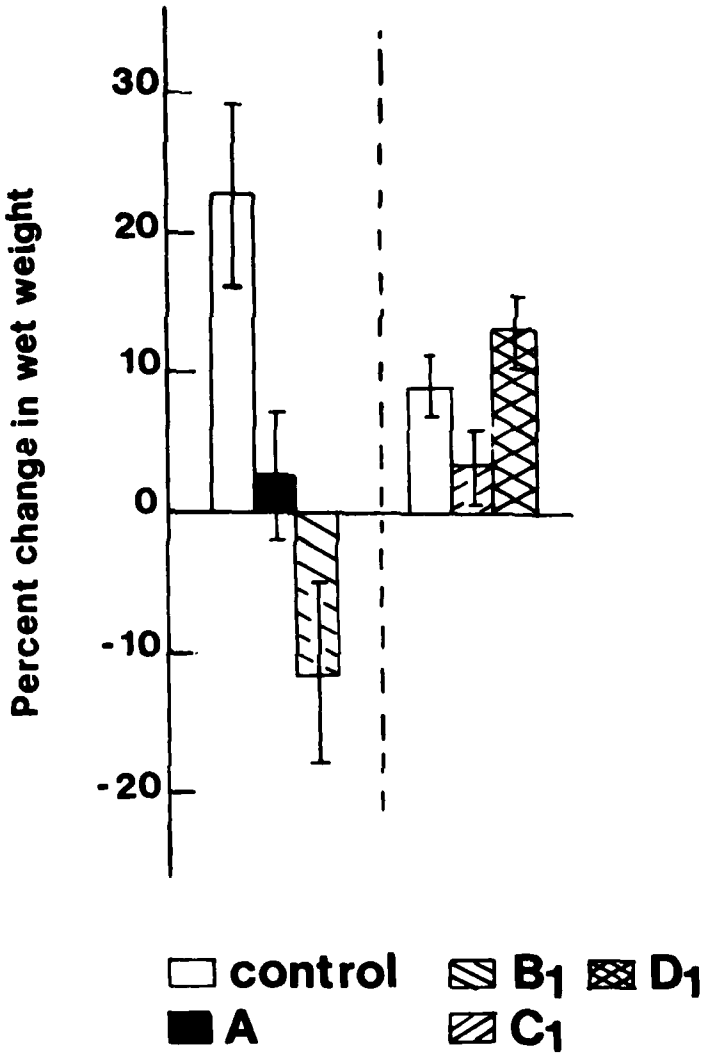


Figure 6. Growth of *Hydrilla verticillata*, measured by percent change in wet weight, in the presence of Lake Starvation sediment extracts (after Dooris and Martin (1980); vertical bars indicate ± 1 SD)

Separation of hydrilla-
inhibiting fraction by HPLC

28. Initial separation by Sep-Pak cartridges was necessary because some material in the hydrilla-inhibiting fraction was strongly adsorbed on the packing used in reverse-phase chromatography. Thus, a preliminary separation removed the very strongly adsorbing material, which constituted a relatively small portion of the total organic content (estimated to be less than 10 percent based upon analysis of organic carbon content before and after adsorption and elution).

29. A number of fractions were evident based upon the information presented in Table 3 and in the chromatograms (Figures 7 and 8). Probably over a dozen fractions could eventually be separated and recognized. It must be emphasized that these are the materials that absorb at 254 nm (fixed wavelength detector).

30. The fractionation conditions involved a solvent gradient in which a weak solvent (methanol) and a strong solvent (water) are used to remove the adsorbed material from a column. In reverse-phase chromatography, the weaker solvent (solvent A) is used first and then the stronger one (solvent B) is added. Several patterns are possible with respect to how the mixture of the weak solvent and the strong solvent is varied during the course of the separation. These are illustrated in Figure 9 where the pattern m is indicated. These studies suggest that a gradient pattern of $m = 3$ is superior to a linear gradient ($m = 1$) for effecting separation of the individual peaks.

31. Individual fractions were collected, but owing to the complexity of the mixture, it must be recognized that the individual fractions may still contain mixtures of substances. The chromatogram for the material adsorbed on a Sep-Pak cartridge and eluted with methanol is illustrated in Figure 8; five fractions were collected. These fractions were based upon the major components illustrated and others that appeared when a more sensitive scale was used that magnified the minor peaks.

32. The fractions were tested for activity against *C. reinhardii*

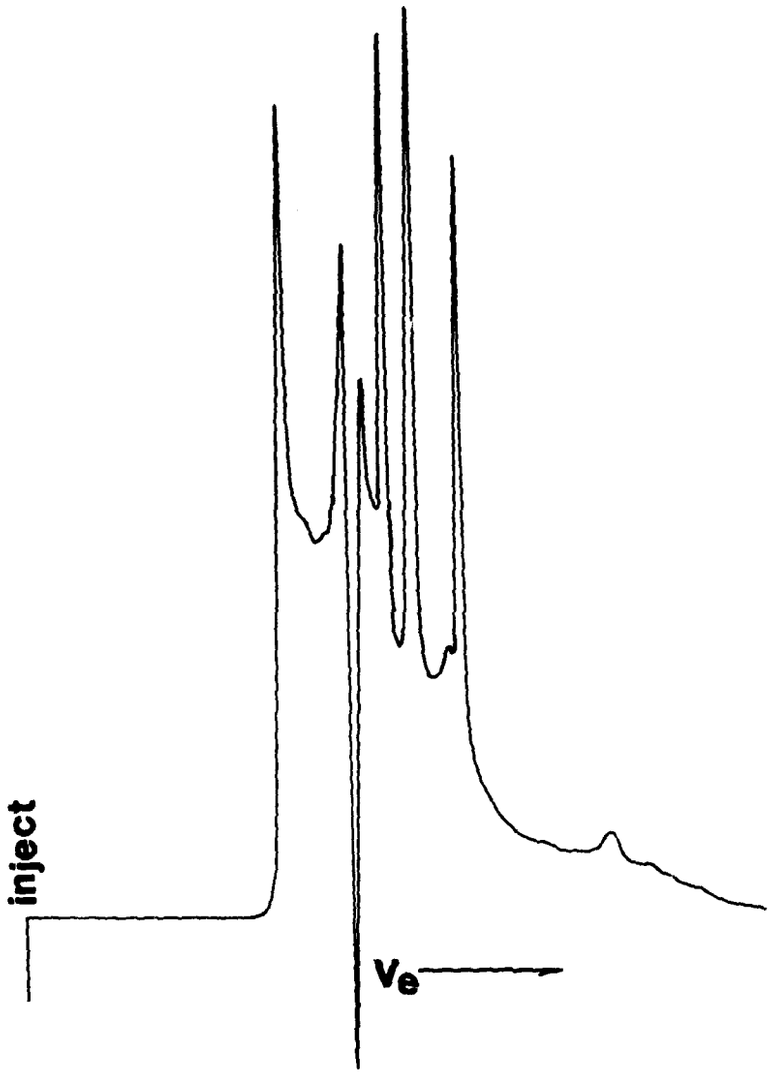


Figure 7. HPLC chromatogram showing separation of fraction B_1 (Lake Starvation sediment extract; see Figure 3) using gradient (program 3, Table 3) and solvent system ranging from 0 percent to 25 percent water in a water-methanol mixture during a 20-min period. Here V_e is the volume of solvent eluted

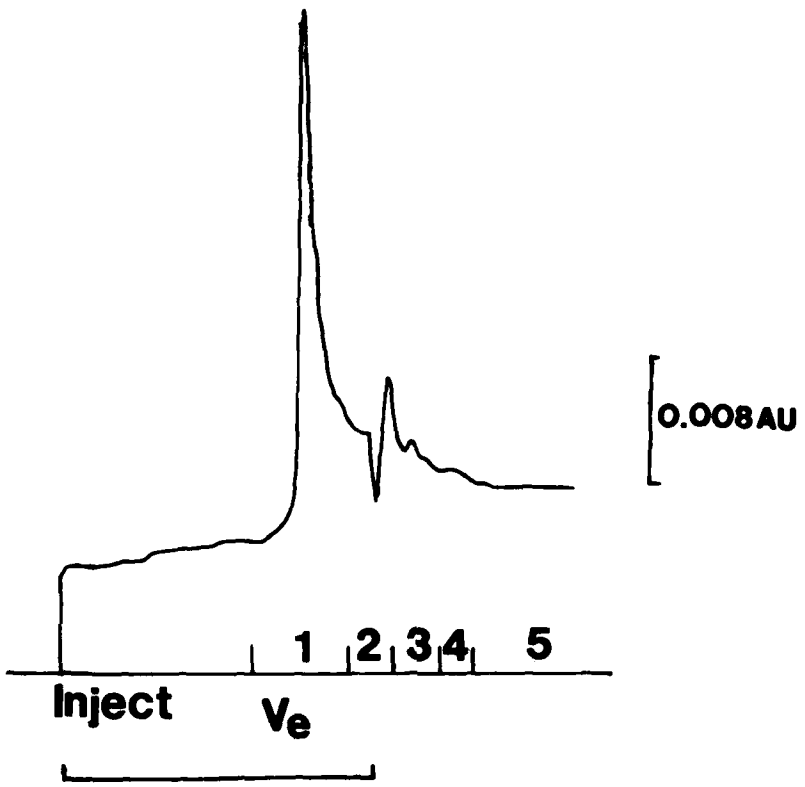


Figure 8. HPLC chromatogram showing separation of fraction B_1 (Lake Starvation sediment extract; see Figure 3) following preliminary adsorption on a Sep-Pak C-18 cartridge and elution with methanol. Fractions collected are indicated by the numbers; horizontal scale is 10 min; vertical scale is 0.008 AU (absorbance units) (Conditions: chart speed, 0.56 cm/min; flow rate, 0.3 ml/min; initial solvent, 95 percent methanol-water; final solvent, 70 percent methanol-water)

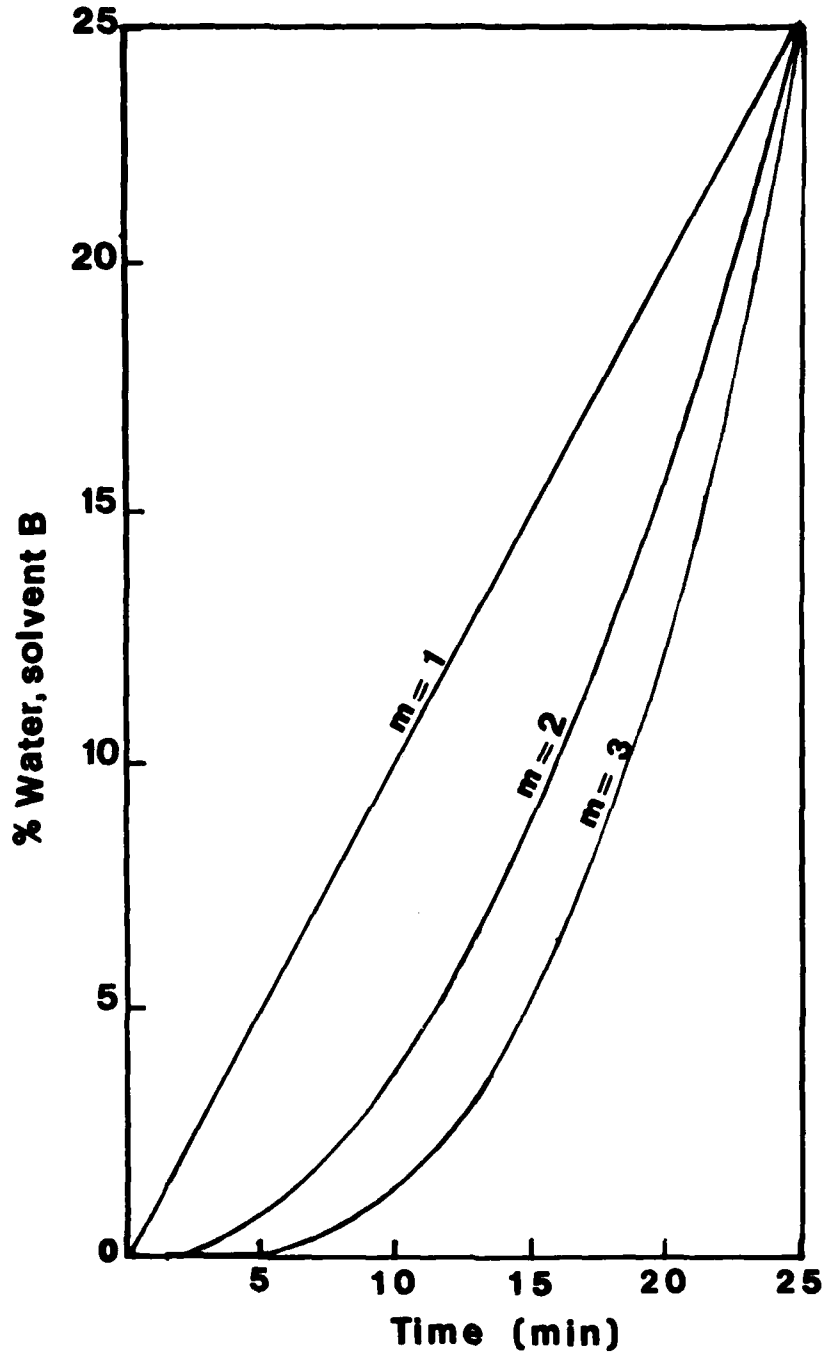


Figure 9. Schematic representation of solvent gradient patterns available for use in HPLC. The selected exponent m is indicated; an exponent setting of m = 1 provides a linear gradient. Here, A is methanol and B is water

and the results are illustrated in Figure 10. Two fractions in the test solutions were active (fractions 3 and 4, as identified in Figure 8). The concentration of individual fractions is slight (total quantity for a 10-µl injection = 8 µg). The fact remains, however, that an effect can be discerned. In addition, control samples were obtained by conducting the chromatographic run, using the appropriate solvents, and collecting fractions at appropriate intervals of time; no samples were injected in the control runs.

33. In addition, the results in Figure 10 may be compared with the ultraviolet spectra (Figure 11), which were obtained for fractions combined from a number of runs, ca. ten.

34. It should also be noted that the initial solvent was not pure methanol, but a 95 percent methanol-5 percent water mixture. With this solvent mixture, many problems encountered using pure methanol did not occur. For example, during the summer, the use of pure methanol for initial elution produced bubbles when the first amounts of water were later added by the solvent gradient apparatus. There is a slight deviation from ideal solution behavior when the first amount of water is introduced since the volumes of water and methanol are not additive; the bubbles that form in the apparatus appear as a strongly absorbing peak on the recorder, and the individual run is worthless. Thus, using the 95 percent/5 percent (v/v) methanol-water mixture, this problem was overcome.

35. The active fraction was separated on the basis of nominal molecular weight, which is based upon an assumed relationship between molecular size and molecular weight. Thus, the nominal molecular weight, based upon ultrafiltration data, would be between 1,000 and 10,000 daltons.

36. The limitations of this estimate are evident, and it must be recognized that smaller, active molecules could be associated with the active fraction. Indeed, the smaller molecules could be active in the inhibition of hydrilla growth.

37. A better estimate of the molecular weight of a significant fraction or a fragment of the active component has come from analysis

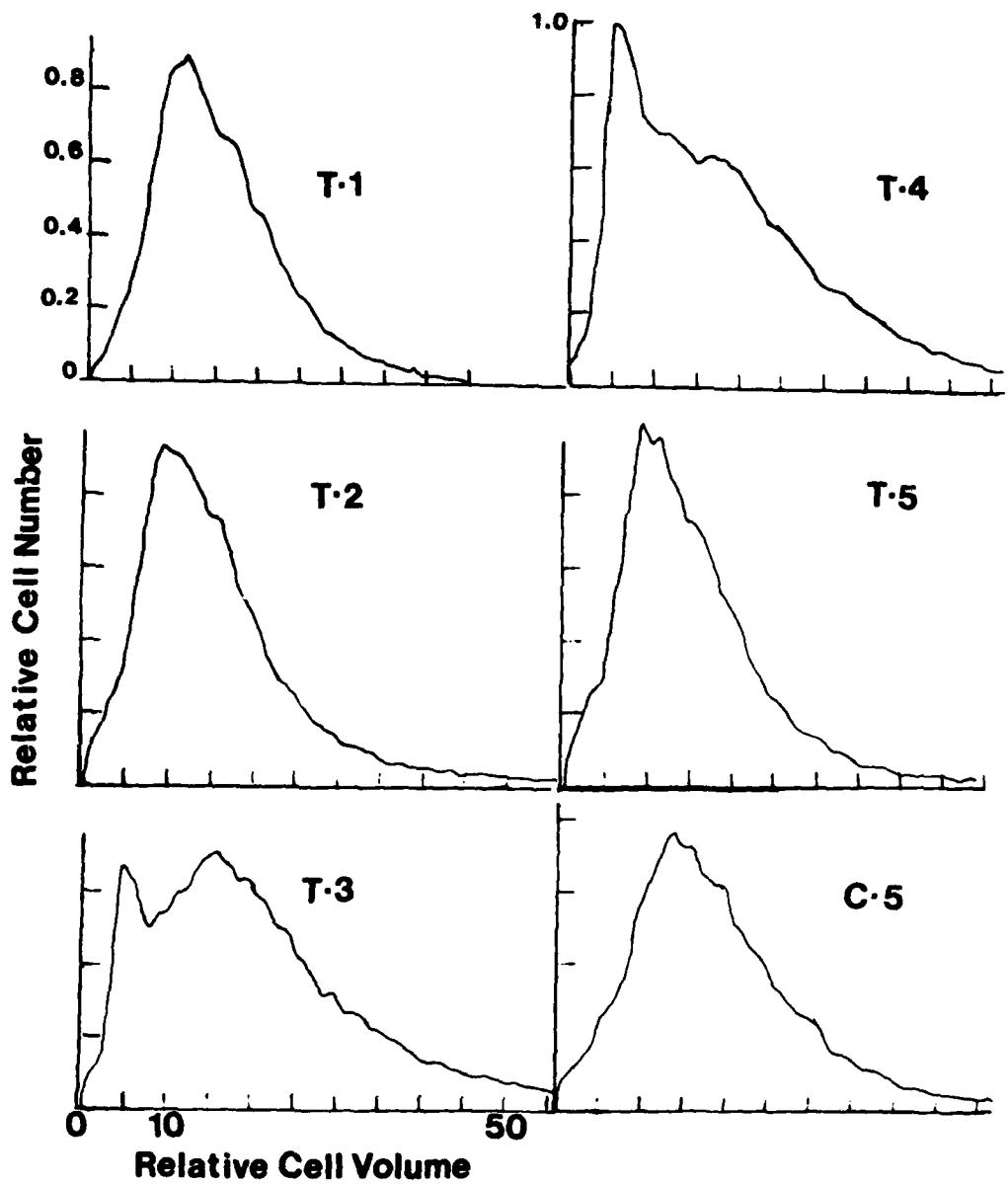


Figure 10. Relative cell numbers as a function of relative cell volume for the interaction of inhibitor fractions 1-4 with *C. reinhardii* following 48 hr incubation at 25°C. Identity of test fractions (T-1 to T-5) is given in Figure 8 (Fraction C-5 is the control)

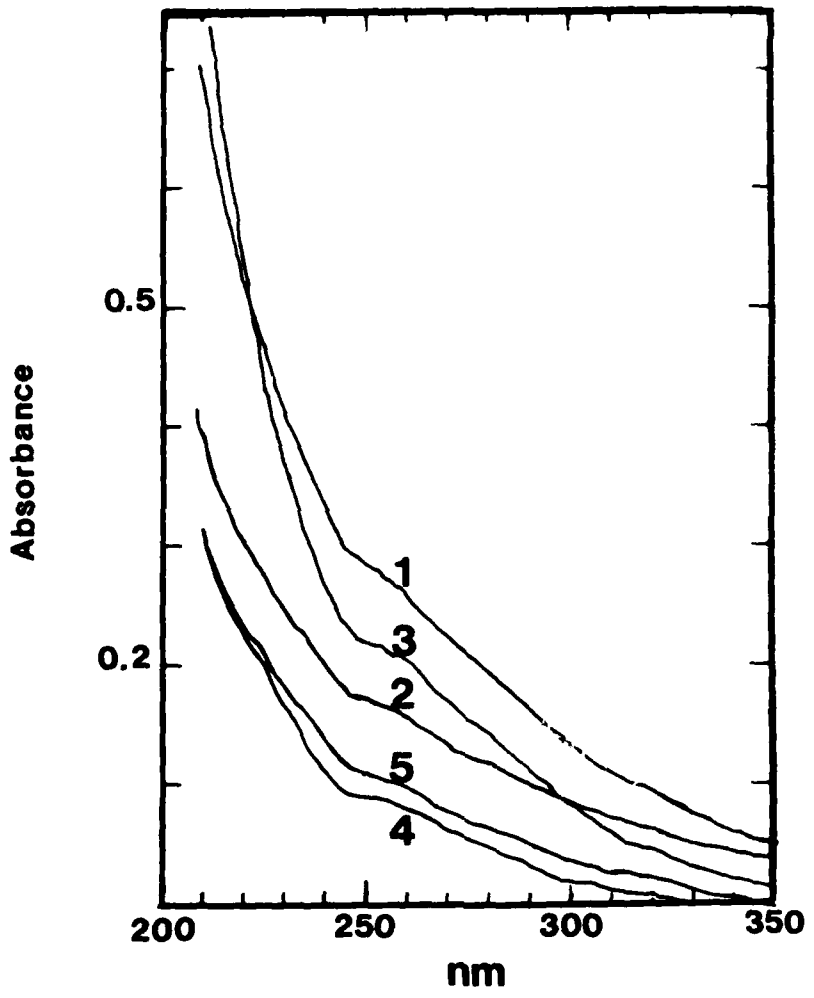


Figure 11. Ultraviolet spectra of purified components 1-5 (see Figure 8)

of purified components. The fractions obtained through HPLC were tested against *C. reinhardii* and the active fractions were submitted to mass spectrometry. The maximum observed molecular weight was about 460 daltons (461 and 459 for two runs, line 1 Table 6). The data do show a fair degree of consistency from one run to another. A molecular ion peak is found at about 460, and the maximum relative intensity was found at 133 (line 13) for four separate runs (two of which are presented in Table 6).

38. Possible molecular formulas can be calculated for each of the observed masses in the mass spectrum (Table 6). With chemical ionization mass spectrometry, in contrast with electron impact mass spectrometry, the most abundant ion is not the molecular ion M^+. From the chemical formulas, it is often possible to determine the structure from the fragmentation, often on the basis of comparison with known compounds.

39. The data in Table 6 are useful as far as they lead to a limited number of conclusions, including the following:

a. The data in Table 6 and 7 show the absence of significant contamination by a common impurity, dialkyl phthalates, as indicated by the absence of such fragments as $C_8H_4O_4$, $C_{10}H_8O_4$, $C_{16}H_{21}O_4$, and $C_{17}H_{23}O_4$, among others.

b. Some aromatic molecules are indicated by rings-plus-double-bonds formula:

rings-plus-double-bonds for $C_xH_yN_zO_n$ = $X-1/2y + 1/2z + 1$ (1)

For example, for $C_{29}H_{47}O_4$ (line 1, Table 6), the number of rings-plus-double-bonds would total about 8. For $C_{26}H_{43}O_2$ (line 3, Table 6), the rings-plus-double-bonds total is 5 to 6, and the two entities (line 1 vs. line 3) differ by the loss of a fragment ($C_3H_4O_2$ or $C_3H_5O_2$) which contained a double-bond and which could be CH_2CH_2COOH or CH_3CH_2COO.

c. Other fragments recognized are CH (line 9 vs. line 11), CH_3 (line 13 vs. line 15), and $C_4H_{10}O_2$ (line 11 vs. line 8), which is an unsaturated alcohol fragment.

40. Other structural information comes from the infrared spectra which have strong absorbances at 3600 to 3630 cm^{-1}, 3100 to 3300 cm^{-1}, 1650 to 1500 cm^{-1}, and 1300 to 1080 cm^{-1}, all of which are consistent with the existence of aromatic, possibly phenolic moieties.

41. In addition, it was found that the inhibitory fraction was

associated with the anionic portion of the active fraction (Dooris and Martin 1980). When the active fraction, B_1, was passed over a cation exchange resin (Chelex-100), no loss in activity occurred. When the material was adsorbed on an anion exchange column, the eluant had neither color nor activity.

Studies of materials possibly related to the Lake Starvation extracts

42. Previous studies have demonstrated that commercially available humic acid, a soil organic acid fraction, can provide extracts that would inhibit the growth of hydrilla (Dooris and Martin 1980).

43. It was hoped that the low water solubility of commercial humic acid could be overcome using the sodium salt of humic acid. In fact, the sodium salt is very soluble, but the extracts did not show the same degree of activity that was observed for the lake extracts. It was possible, however, to extract the sodium humate, then convert the sodium humate to "humic acid" by means of a cation-exchange column (H^+-form, with replaceable H^+). Thus, a hydrilla-inhibiting material was obtained from a commercial source.

Conclusions

44. It has been demonstrated that a material that inhibits the growth of *Hydrilla verticillata* can be obtained from the aqueous extracts of Lake Starvation sediment.

45. The inhibiting character of the extracts did not appear to be the result of manganese toxicity (Table 1). In addition, nickel and cadmium levels in the extracts were also below reasonable detection limits (<0.05 ppm) and the iron levels in the extracts did not appear to account for inhibition of hydrilla growth.

46. It was possible to separate the active fraction by means of ultrafiltration using sized membrane filters. The active fraction, obtained from ultrafiltration, can be further fractionated into components, some of which are active against the test organism *Chlamydomonas reinhardii*. The active material also inhibited hydrilla in contact with

sediment (50 percent peat, 50 percent sand).

47. It seems evident that the inability of hydrilla to thrive in Lake Starvation is, in part, the result of naturally occurring materials that are extracted from the sediment, though the mechanism of production of these materials is presently unknown.

48. Commercially available humic acid can also be extracted to produce a hydrilla-inhibiting fraction at a level of about 0.4 ppm as dissolved organic carbon.

49. Useful structural information has been obtained from mass spectrometry and infrared spectra of the purified components of the active fraction.

Recommendations

50. Large-scale separation and purification of the active components are needed. The general separation is not a serious problem, but the purification by HPLC requires time and patience.

51. Studies of the mass spectra need to continue, and, in particular, whether or not the fragments can serve as models for useful herbicides needs to be considered.

52. It has been demonstrated that humic acid, as formerly available commercially, can be extracted to form a useful inhibitor. As it happens, the former source of supply is closed, and the by-product is now a sodium humate, available from Germany through an American firm. It is believed that conversion is feasible, as has been demonstrated.

53. A large sample of purified material should be considered for screening using the continuous-flow testing technique in a Corps-supported laboratory.

54. It is believed that it is possible to "fingerprint" the hydrilla-inhibiting material through the use of HPLC analysis, coupled with suitable bioassays. Investigations need to include not only interstitial water from Lake Starvation sediment, but also interstitial water from other lakes being monitored by Corps personnel, e.g. Lake Seminole.

55. The mode of action of the hydrilla growth inhibitor needs to be investigated. It seems likely that the material is functioning as a chelating agent, and could be affecting metal ion transport and subsequent processes that depend upon metal-ion concentration. Understanding the mode of action would allow researchers to predict accurately where the inhibitor would work best in natural waterways.

56. The economics of management by natural products need to be considered with respect to reduction of monitoring costs and in comparison with other approaches, e.g., application of herbicides, bio-control, and dredging (Dooris, Ley, and Martin 1982).

References

Ball, D. F. 1964. "Methodology for the Loss-On-Ignition Estimate of Organic Carbon and Organic Matter in Non-Calcareous Soils," Journal of Soil Science, Vol 15, pp 84-92.

Chen, K. Y., et al. 1976. "Research Study on the Effect of Dispersion, Settling, and Resedimentation on Migration of Chemical Constitutents During Open-Water Disposal of Dredged Materials," Contract Report D-76-1, U. S. Army Engineer Waterways Experiment Station, CE, Vicksburg, Miss.

Cooley, T. N., Dooris, P. M., and Martin, D. F. 1980. "Aeration as a Tool to Improve Water Quality and Reduce the Growth of Hydrilla," Water Research, Vol 14, pp 485-489.

Dooris, P. M., Ley, V., and Martin, D. F. 1982. "Laboratory Experiments as an Aid to Lake Drawdown-Decision Making. Sawgrass Lake as a Case History," Water Resources Bulletin, In press.

Dooris, P. M., and Martin, D. F. 1979. "Ground-Water Induced Changes in Lake Chemistry," Ground Water, Vol 17, pp 324-327.

Dooris, P. M., and Martin, D. F. 1980. "Growth Inhibition of *Hydrilla verticillata* by Selected Lake Sediment Extracts," Water Research Bulletin, Vol 16, pp 112-117.

Dooris, P. M., and Moresi, R. J. 1975. "Evaluation of Lake Augmentation Practices in Northwest Hillsborough County, Florida," Technical report prepared for the Southwest Florida Water Management District, Brooksville, Fla.

Jackson, M. L. 1956. Soil Chemical Analysis - Advanced Course (Fifth printing, 1969), Published by the author, Department of Soil Science, University of Wisconsin, Madison, Wis.

Ohad, I., Siekevitz, P., and Palade, G. E. 1967. "Biogenesis of Chloroplast Membranes," Journal of Cell Biology, Vol 35, pp 521-552.

Reid, G. A., Jr., Martin, D. F., and Kim, Y. S. 1975. "Stimulation of Hydrilla growth by $Fe(EDTA)^-$," Journal of Inorganic Nuclear Chemistry, Vol 37, pp 357-358.

Sager, R., and Granick, S. 1953. "Nutritional Studies with *Chlamydomonas reinhardii*," Annals of the New York Academy of Sciences, Vol 56, pp 831-838.

Soil Conservation Service. 1958. "Soil Survey of Hillsborough County, Florida Series 1950," No. 3, United State Department of Agriculture, Washington, D.C.

Steward, K. K., and Elliston, R. A. 1973. "Growth of *Hydrilla* in Solution Culture at Various Nutrient Levels," Florida Scientist, Vol 36, pp 228-233.

Table 1

Selected Analyses of Lake Starvation Sediment and Aqueous Extracts

Samples	Organic Carbon, ppm	Metal Content, ppm Fe	Mn
Dried sediment	26.1×10^4	277±32*	<10*
		556±33**	<10**
Extract A†	762	0.2	< 0.05
A_1	2,380	1.0	< 0.05
B	381		
B_1	210	0.5	< 0.05
C	49		

* Sodium acetate buffer, pH 5, paragraph 13.

** Ammonium acetate buffer pH 2 and 5, paragraph 14, Ni and Cd < 0.05 ppm.

† A is the parent fraction (see Figure 3), A_1 is that portion of the fraction that did not pass through the um-10 membrane; fraction B did pass through this membrane, etc.

Table 2

Comparisons of Carbon Content of Various Extracts

Material	Fraction	Carbon Concentration, ppm Inorganic	Organic
Sediment			26.1×10^4
Aqueous extract of sediment, mean of six samples*	A		762
Aqueous extract of sediment, single sample, fractionated*	A_1**		2380
	B		381
	B_1		210
	C		49
Sediment	A	25	43
Room temperature	A_1	3	97
Extract	B	23	25
	B_1	27	28
	C	27	28
Humic acid	A_1	1	1700
Aqueous	B_1	1	790
Extract**	C	1	300

* Extracted at 120°C, 138 kPa, 20 min.

** Subscript denotes sample that did not pass through a given membrane (see Figure 3 and Table 1).

Table 3

Analysis of Lake Starvation Sediment with HPLC and a Gradient Attachment

Program number	Length of run, min	Gradient Characteristics			Number of peaks observed in chromatogram
		Initial percent of solvent B *	Final percent of solvent B *	Exponent n used **	
1	20	0	25	3	8
2	20	5	25	3	9
3	20	10	30	3	9
4	20	20	40	4	8
5	20	20	40	3	7
6	20	20	40	2	7
7	20	20	40	1	poor resolution
8	20	0	100	1	2
9	40	10	80	1	6
10	20	20	50	1	3
11	20	10	40	1	8

* Solvent A, methanol; solvent B, water.

** See Figure 8.

Table 4

Culture Medium for *Chlamydomonas reinhardii*

Stock Solution A			Stock Solution B	
Reagent	grams/litre	Concentration mM	Reagent	Concentration mg/100 ml
$K_2HPO_4 \cdot 3H_2O$	1.31	72.5	HBO_3	100
KH_2PO_4	1.00	72.3	$ZnSO_4 \cdot 7H_2O$	100
NH_4NO_3	3.00	37.5	$MnSO_4 \cdot H_2O$	40
Citric acid	0.10	0.47	$CoCl_2 \cdot 6H_2O$	20
Sodium citrate	5.00	17	$Na_2MoO_4 \cdot 2H_2O$	20
$CaCl_2$	0.40	2.7	$CuSO_4 \cdot 5H_2O$	6
$MgSO_4 \cdot 7H_2O$	1.50	6.1		
Iron citrate	0.100			

Solution A was autoclaved just after preparation and stored in a refrigerator. Medium was prepared using 100 ml of Stock Solution A, 1 ml of Stock Solution B, and enough distilled water to prepare 1000 ml of solution (Ohad, Siekevitz, and Palade 1967; Sager and Granick 1953).

Table 5

Summary of Counting Experiments for *Chlamydomonas reinhardii* Using a Coulter Counter Model ZB_I

Dilution	Relative Cells	N *	Mean	SD	%CV **
0.1	1	6	29,140	±400	1.3
0.05	0.5	4	17,901	420	2.3
0.02	0.2	4	8,390	32	0.4
0.01	0.1	7	5,053	± 43	0.9
.....					
		6	913	± 26	2.8

* N = number of determinations

** %CV = coefficient of variation = (SD/mean) x 100.

Table 6

Summary of High Resolution Mass Spectra of HPLC-Purified Sample of Hydrilla Inhibitor (Fraction 3)* for Two Runs

	Observed Mass	Relative Intensity	Possible Formula	Observed Mass	Relative Intensity	Possible Formula
1.	461.0168	1.8	$(C_{29}H_{49}O_2)$	459.3483	1.1	$C_{29}H_{47}O_4$
2.	399.3190	2.5	$C_{27}H_{43}O_2$	-	-	-
3.	387.3473	2.7	$C_{23}H_{47}O_4$	387.3280	5.1	$C_{26}H_{43}O_2$
4.	386.3682	2.3	$C_{24}H_{50}O_3$ $C_{23/1}H_{49}O_3$** $C_{23}H_{48}O_3N$	386.3342	14.1	$C_{23}H_{46}O_4$ $C_{22/1}H_{45}O_4$
5.	386.3325	17.9	$C_{23}H_{46}O_4$	-	-	-
6.	385.3315	51.2	$C_{23}H_{45}O_4$	385.3312	38.1	$C_{23}H_{45}O_4$
7.	-	-	-	372.3191	3.5	$C_{22}H_{44}O_4$

(Continued)

* Chemical ionization, 200°C, 8 KV, 70 EV, CH_4 gas; see Figures 8 and 10 for additional description of Fraction 3.

** $C_{20/1}$ means isotope ratio, i.e., $^{12}C/^{13}C$.

Table 6 (Continued)

	Observed Mass	Relative Intensity	Possible Formula	Observed Mass	Relative Intensity	Possible Formula
8.	371.3004	8.8	$C_{25}H_{39}O_2$ $C_{20/1}H_{40}O_4N$**	371.3172	9.2	$C_{22}H_{43}O_4$
9.	151.0350	15.8	$C_{7/1}H_6O_3$ $C_8H_7O_3$	151.0153	5.9	$(C_{11}H_3O)$
10.	149.0296	15.4	$C_{12}H_5O$	149.0433	2.6	$(C_{12}H_5)$
11.	134.0573	11.5	$C_5H_{10}O_4$	134.0223	13.5	$C_7H_4O_2N$ $C_4H_6O_5$ $C_8H_6O_2N$
12.	134.0455	9.0	$C_8H_6O_2$	-	-	-
13.	133.0479	100.0	$C_5H_9O_4$	133.0169	100	$C_4H_5O_5$ $C_7H_3O_2N$
14.	119.0274	7.6	$C_4H_7O_4$ C_7H_6O C_7H_5ON	-	-	--
15.	-	-	-	118.8986	50.6	-
16.	-	-	-	112.8803	4.4	-
17.	-	-	-	99.8094	9.6	-
18.	97.1081	15.0	C_7H_{13}	-	-	-

In accordance with letter from DAEN-RDC, DAEN-ASI dated 22 July 1977, Subject: Facsimile Catalog Cards for Laboratory Technical Publications, a facsimile catalog card in Library of Congress MARC format is reproduced below.

Martin, Dean F.
Study of a naturally occurring hydrilla inhibitor / by Dean F. Martin (Chemical and Environmental Management Center, University of South Florida). -- Vicksburg, Miss. : U.S. Army Engineer Waterways Experiment Station ; Springfield, Va. : available from NTIS, 1983.
29, [7] : ill. ; 27 cm. -- (Miscellaneous paper ; A-83-2)
Cover title.
"March 1983."
Final report.
"Prepared for Office, Chief of Engineers, U.S. Army under Contract No. DACW39-80-C-0066."
"Monitored by Environmental Laboratory, U.S. Army Engineer Waterways Experiment Station."
At head of title: Aquatic Plant Control Research Program.
Bibliography: p. 28-29.

1. Aquatic weeds. 2. Hydrilla. 3. Lake Starvation (Fla.) 4. Sedimentation and deposition. 5. Weed

Martin, Dean F.
Study of a naturally occurring hydrilla inhibitor : ... 1983.
(Card 2)

control. I. United States. Army. Corps of Engineers. Office of the Chief of Engineers. II. University of South Florida. III. Aquatic Plant Control Research Program. IV. Title V. Series: Miscellaneous paper (U.S. Army Engineer Waterways Experiment Station) ; A-83-2.
TA7.W34m no.A-83-2


D
FI
7